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Liposomal antibiotic delivery system towards Gram-negative bacteria

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Chemical Engineering B.Sc.

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για την απόκτηση Μεταπτυχιακού Διπλώματος Ειδίκευσης στα ‘Νανοφάρμακα για την χορήγηση φαρμάκων (“Nanomedicines for Drug Delivery” – NANOMED)’

ΠΑΤΡΑ 2019

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ΚΛΕΠΕΤΣΑΝΗΣ ΠΑΥΛΟΣ, Αναπληρωτής Καθηγητής - Επιβλέπων

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ΠΕΡΙΛΗΨΗ

Η αύξηση της ανθεκτικότητας των μολύνσεων με Gram-αρνητικά βακτηρίδια σε πολλά φάρμακα, αποτελεί σημαντικό ζήτημα για τη δημόσια υγεία. Ο κύριος μηχανισμός αντοχής στα παθογόνα προκύπτει από τη χαμηλή διαπερατότητα της εξωτερικής μεμβράνης των αρνητικών κατά Gram βακτηρίων στους περισσότερους αντιβακτηριακούς παράγοντες που είναι σήμερα διαθέσιμοι.

Σκοπός αυτής της μελέτης ήταν η εκτίμηση της δυνατότητας χρήσης λιποσωμικών μορφών για να επαναχρησιμοποιηθούν τα σημερινά αντιβιοτικά, ή για να σχεδιαστούν νέες θεραπείες συνδυασμού με αντιβακτηριακούς παράγοντες. Στην παρούσα μελέτη περιγράφεται ένας νέος τρόπος ενίσχυσης της αντιβακτηριακής ενεργότητας με επιλεκτική στόχευση του E.coli και θεραπεία συνδυασμού με ένα άλλο αντιβιοτικό, την τετρακυκλίνη. Παρασκευάστηκαν λιποσώματα με φθορίζουσα σήμανση, με τη μέθοδο ενυδάτωσης λεπτή μεμβράνης και η αντιβακτηριακή ενεργότητα των λιποσωμάτων αξιολογήθηκε με μέτρηση οπτικής πυκνότητας και προσδιορισμό χρόνου θανάτου. Το μέσο μέγεθος των λιποσωμάτων κολιστίνης ήταν 149,2 ± 16,45 nm με 0,128 PDI που καταδεικνύει στενή κατανομή μεγέθους. Τα αποτελέσματά μας έδειξαν ότι τα λιποσώματα που περιέχουν κολιστίνη ενισχύουν την αντιβακτηριακή δραστικότητα σε σύγκριση με την ελεύθερη κολιστίνη, μέσω επίδρασης συμπύκνωσης η οποία διατηρεί υψηλή τοπική συγκέντρωση φαρμάκου στην εξωτερική μεμβράνη του E. Coli οδηγώντας σε διαταραχή της εξωτερικής μεμβράνης και στη συνέχεια θανάτωση των βακτηριδίων.

Η συγχορήγηση τετρακυκλίνης και κολιστίνης σε λιποσώματα έδειξε ότι η αντιβακτηριακή δράση της τετρακυκλίνης αποκαθίσταται όταν συνδυάζεται με κολιστίνη σε χαμηλότερη συγκέντρωση. Ως εκ τούτου, η αντιμικροβιακή αντοχή της τετρακυκλίνης μπορεί να μετριαστεί με αυτή την προσέγγιση αξιοποιώντας την δράση στόχευσης κολιστίνης. Αυτή η μελέτη δείχνει μια νέα οδό για τη χορήγηση αντιβιοτικών ειδικά με την αύξηση της θεραπευτικής αποτελεσματικότητάς τους, ελαχιστοποιώντας τη συστηματική έκθεση

και ενισχύοντας τη συγκράτηση στη θέση της λοίμωξης η οποία αντιπροσωπεύει μια πολλά υποσχόμενη προσέγγιση για την ανάπτυξη στρατηγικών χορήγησης γαστρεντερικών λοιμώξεων.

ABSTRACT

The rise of multidrug resistance in Gram-negative bacteria infections is a major public health issue. The main mechanism of resistance in pathogens is emerging from low permeability of Gram-negative bacteria outer membrane to most of the antibacterial agents that are currently available. The aim of this study was assessing the potential of liposomal formulation strategies to repurpose current antibiotics or design new combination therapy with antibacterial agents.

This study describes a novel way to enhance antibacterial activity by selective targeting of *E.coli* and combination therapy with another antibiotic, Active Ingredient. Fluorescently labelled liposomes were prepared by thin film hydration method and antibacterial activity of liposomes were evaluated by optical density measurement and time-kill assay. The average particle size of colistin encapsulated liposomes were 149.2 ± 16.45 nm with 0.128 PDI demonstrating narrow size distribution. Our results demonstrated that colistin encapsulated liposomes enhance antibacterial activity compared to free colistin via concentration effect which is elucidated as maintaining high local drug concentration at the outer membrane of *E.coli* leading to outer membrane disruption and subsequently killing of the bacteria.

Co-administration of Active Ingredient and colistin in liposomes showed that antibacterial activity of Active Ingredient is restored when in combination with colistin in lower concentration. Hence antimicrobial resistance of Active Ingredient can be mitigated with this approach by exploiting colistin targeting effect. This study establishes a novel pathway to deliver antibiotics specifically by increasing their therapeutic efficiency, minimising systemic exposure and enhancing retention at the site of infection which represents a promising approach for developing delivery strategies for gastrointestinal infections.

NANOMED EMJMD

Nanomedicine for drug delivery

6 months traineeship report

Years 2017/2019

Liposomal Antibiotic Delivery System towards Gram-Negative Bacteria

This report is confidential

Date of defence: 10/07/2019

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Co-funded by the Erasmus+ Programme of the European Union

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ACKNOWLEDGEMENTS

The realisation of this research project was possible thanks to the multidisciplinary collaboration between the School of Pharmacy and School of Life Sciences. I therefore thank all those who contributed to the realisation of this project, in particular my supervisors Prof. Snow Stolnik (School of Pharmacy, University of Nottingham), Dr. Rosa Catania (EPSRC Postdoctoral Fellow at University of Nottingham) and Dr. Alan Huett (School of Life Sciences, University of Nottingham) for their help in the experimental design, lab expriments and the data analysis of the work here presented.

ABSTRACT

The rise of multidrug resistance in Gram-negative bacteria infections is a major public health issue. The main mechanism of resistance in pathogens is emerging from low permeability of Gram-negative bacteria outer membrane to most of the antibacterial agents that are currently available. The aim of this study was assessing the potential of liposomal formulation strategies to repurpose current antibiotics or design new combination therapy with antibacterial agents. This study describes a novel way to enhance antibacterial activity by selective targeting of *E. coli* and combination therapy with another antibiotic, Active Ingredient. Fluorescently labelled liposomes were prepared by thin film hydration method and antibacterial activity of liposomes were evaluated by optical density measurement and time-kill assay. Particle size of the liposomes colistin encapsulated liposomes were 120-150 nm size range with less than 0.2 PDI demonstrating narrow size distribution. Our results demonstrated that colistin encapsulated liposomes enhance antibacterial activity compared to free colistin whcih may be attributed to a concentration effect – liposomes maintaining high local drug concentration at the outer membrane of *E. coli* leading to outer membrane disruption and subsequently killing of the bacteria. This study establishes a novel pathway to deliver antibiotics specifically by increasing their therapeutic efficiency, minimising systemic exposure and enhancing retention at the site of infection which represents a promising approach for developing delivery strategies for gastrointestinal infections.

Keywords: Liposome, Colistin, Lipopolysaccharide, Gram-negative bacteria, Antimicrobial resistance, Minimum inhibitory concentration (MIC)

TABLE OF CONTENTS

Abbreviations

LPS: Lipopolysaccharide

OM: Outer membrane

SM: Sphingomyelin

SUV: Small unilamellar vesicle

LUV: Large unilamellar vesicle

MLV: Multilamellar vesicle

MH Broth: Mueller-Hinton Broth

1. Introduction

The rise of antibiotics resistance is a major public health issue around the world. The increasing burden of multidrug resistant Gram-negative infection is particularly concerning as such bacteria are demonstrating resistance to nearly all currently licensed therapies (1). Hence, the growing burden of multidrug resistance in Gram-negative bacteria demands not just to discover novel antibacterial agent but also to improve the efficiacy of current therapies (2). The latter can be achieved by using advanced drug delivery systems. Various advanced drug delivery formulations arising from nanotechnology (polymeric nanoparticles, liposomes, solid lipid nanoparticles, nanocapsules, micelles etc) have been extensively studied. Nano drug delivery systems are also emerging in the area of antibiotic therapies. Recent works (e.g. liposomal Active Ingredient, AmBiosome) demonstrates that encapsulation of antibiotics into nanoparticles can achieve reduced toxicity and improved biodistribution (2). Design of advanced antibiotic formulations is beginning to demonstrate future potential. Nanomedicines have several advantages like reducing the toxicity of antibiotic agents, having a versatile structure enabling to modify physicochemical properties, enhancing the antibacterial activity, selective targeting by nanoparticle surface modifications, reducing dosing frequency and modification of pharmacokinetic properties and biodistribution of the antibacterial agent (3). Recent research demonstrated that liposomes containing linolenic acid have bactericidal properties against Gram-negative *H. Pylori*. This effect is emerging from the rapid fusion the nanoformulation with bacteria membrane (4).

The aim of this work is assessing the potential of liposomal formulation strategies to repurpose current antibiotics or design a new combination therapy with antibacterial agents and their delivery formulations for gastrointestinal infections by targeting the Gram-negative bacteria and enhancement of antibacterial activity by encapsulation in liposomes.

The diseases caused by Gram-negative bacteria in the gastrointestinal tract are salmonellosis, diarrhoea, gastric ulcer and enterocolitis. Gram-negative bacteria strains responsible for such diseases are mainly, *Escherichia coli (E. coli)*, *Helicobacter pylori (H. Pylori)*, *Salmonella typhimurim, Shigella*, *Pseudomonas aeruginosa* - causing cystic fibrosis.

Eradication of Gram-negative bacteria in the gastrointestinal tract by liposomes is a potential strategy for treatment of infections (5). However, there are some limitations due to liposome instability in harsh environment of gastrointestinal tract, bile salts can interact with liposomes leading to disruption of liposome integrity or destabilisation (5). According to current studies, polymer coating (chitosan, pectin etc) on liposomes or the use of phospholipids with phase transition temperature higher than 37°C in formulation could

improve the liposome stability in the gastrointestinal (GI) tract. Because liposomes containing phospholipids with phase transition temperatures below 37 °C are completely disrupted by bile salts, but this effect is less pronounced for those with phase transition temperature higher than 37 °C (6). Despite this, oral delivery of liposomes is a challenging task.

The mechanisms of antimicrobial resistance in Gram-negative bacteria often involve modification of outer membrane (8). There are also other mechanisms such as efflux pumps, drug inactivation and target alteration in bacteria (7). In this work, we investigated the efficiacy of liposome formulations on different lipopolysaccharide presenting *E. coli* strains. Various strategies have been studied to treat multidrug resistant Gram-negative bacteria infections including targeting the Gram-negative bacteria outer membrane; neutralisation of lipopolysaccharide; inhibition of bacterial efflux pumps and prevention of protein folding (8). The unique and complex structure of the Gram-negative bacteria membrane acts as a significant permeability barrier, reducing sufficient drug levels at the required sites of action (7)(8).

The Gram-negative cell wall consists of an inner membrane of phosphatidylethanolamine, phosphatidylglycerol and cardiolipin as principal phospholipid components, together with an asymmetric outer membrane composed of a phospholipid in the inner side, and lipopolysaccharides in the outer side (9). Lipopolysaccharide (LPS) of Gram-negative bacteria is a permeability barrier essential for bacteria viability (7).

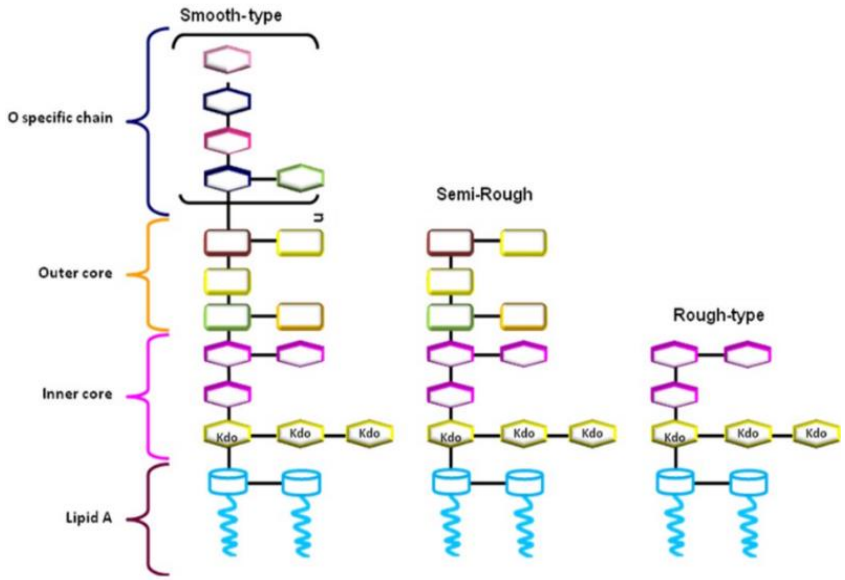


Figure 1 Types of LPS structure (11)

LPS structure comprises (i) lipid A, the hydrophobic moiety that anchors LPS to the outer leaflet of the outer membrane; (ii) core oligosaccharide, which together with lipid A, contributes to maintain the integrity of the outer membrane; and (iii) O antigen, which is connected to the core and consists of a polymer made of repeating oligosaccharide units (10). Strong intermolecular interactions of LPS with divalent cations (*e.g.*, Mg^{2+} and Ca^{2+}) are considered the primary mechanism of enhancing the outer membrane stability and limiting the permeation of external agents such as lipophilic antibiotic compounds (10).

The presence or absence of O-antigen chains determines whether LPS is considered rough or smooth (11). As shown in Fig. 1, smooth LPS is characterised by full-length O-antigen, whereas the absence or reduction of O-antigen chains are typical of the LPS rough. Bacteria with rough LPS usually have more penetrable cell membranes to hydrophobic antibiotics, since rough LPS is more hydrophobic. The O - antigen is recognised by the innate immune response and participates in complement activation and in the inhibition of the formulation of the complex that attacks the membrane (11).

Colistin (polymyxin E) is a decades-old antibiotic used for treating infections caused by Gram-negative multidrug-resistant bacteria (12). However, the use of colistin became limited due to its nephrotoxicity which led to its withdrawal from clinical use during the 1960s until it emerged again as last resort antibiotic due to lack of new antimicrobial agents against multidrug-resistant Gram-negative bacteria infections (13)(14). Colistin is an amphiphilic, high molecular weight drug molecule containing a cationic polypeptide ring and a fatty acid chain bound to the polypeptide via covalent bond (15)(16). Colistin sulfate is freely soluble in water; insoluble in acetone and in ether (15). Colistin is bacteria membrane disruptor and increases the permeability of the outer membrane of Gram-negative bacteria (17). However detailed colistin mechanism of action on Gram-negative bacteria is not well known, there are proposed mechanisms according to recent studies (17). There are electrostatic and hydrophobic interactions involved between colistin and lipopolysaccharide leading to destabilisation of the outer membrane hence membrane permeability increases, colistin access to cytoplasmic membrane then causes lysis and bacteria death. Hydrophobic and hydrophilic regions of colistin interact with the cytoplasmic membrane with a detergent like mechanism (18).

As shown in Fig. 2, the electrostatic interaction between the positively charged polypeptide ring and the negatively charged lipid A phosphates is believed to displace divalent cations (Ca^{2+} and $Mg^{2+)}$ that normally function to bridge and stabilise the outer membrane (18)(19). Sabnis *et al.* (20) relates colistin mechanism of action to a process called "de novo lipopolysaccharide (LPS) biosynthesis". According to this process, de novo LPS production and LPS release from the outer membrane is required for colistin activity. In

addition, the lipid tail of colistin leads to severe disruption on outer membrane allowing colistin to reach cytoplasmic membrane (20).

A drug-delivery system that could reduce antibiotic toxicity while increasing the therapeutic efficiency is of great interest, and liposome encapsulated antimicrobial agents can provide these benefits (3) (21). Colloidal carriers like liposomes can enhance penetration and retention of antibiotics on the bacteria cell membrane (21). However, drugs that show both hydrophilic and lipophilic properties may be able to pass the lipid membrane and leak out of the liposomes which leads to long term stability problem (22).

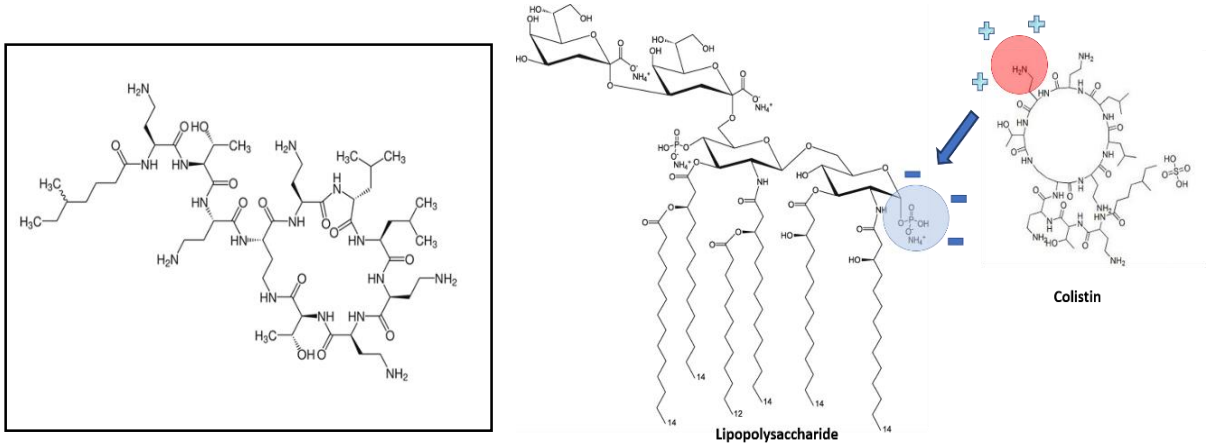


Figure 2 Colistin chemical structure (left) and LPS – colistin interaction (right).

One of the possible mechanism of interaction between liposome and bacteria is fusion. It was recently established that the main driving force for the liposome–bacterial fusion is due to the nature of bacterial membranes (23). There is a correlation between the degree of liposome–bacteria fusion and the percentage of phosphatidylethanolamine (PE) in the bacteria membrane. Some wild type Gram- negative bacteria fusion is not impossible (23).

For this study, phospholipid components were chosen as; Cholesterol, Sphingomyelin (Egg-sphingomyelin) and POPC (1-palmitoyl-2-oleoyl-glycerol-3-phosphocholine) for liposome formulation. Liposome size aimed for this application is in the range of 100 nm. We hypothesised that colistin intercaleled in the phospholipid bilayer for its amphiphilicity, as shown in Fig. 3. In addition, DSPA-Na (1,2- Distearoyl-sn-glycerol-3- phosphate, sodium salt) was used also been used in set of formulations to investigate the binding of Gram-negative bacteria and liposome.

Self-assembly process of phospholipids to form liposomes involves many parameters. Physicochemical properties and production parameters of liposomes are tunable which affects drug loading and antibacterial effect (22). These parameters are; bilayer rigidity,

drug/lipid ratio, liposome size and surface charge, sonication time, evaporation duration, residual organic solvent and amount of evaporated organic solvent, phase transition temperatures of lipids used, mechanical agitation (energy), processing (hydration and formulation) temperature and liposome type (SUV, MLV or LUV) (25)(3).

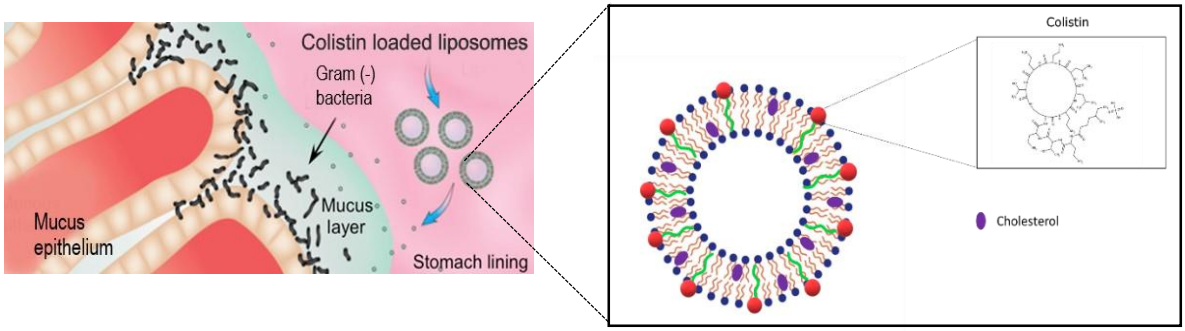
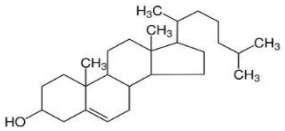
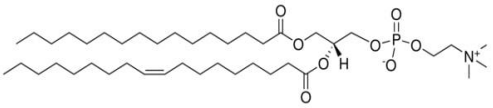
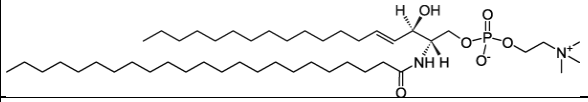
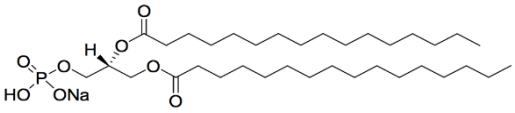


Figure 3 Colistin liposome in gastrointestinal (GI) tract and the structure of colistin loaded liposomes

In this study colistin content in liposomes was quantified by using Ninhydrin colorimetric assay. Development of ninhydrin assay for colistin was based on amine (NH_2) groups present in colistin. The colour change from yellow (colour of ninhydrin agent) to purple occurs during amine group consumption in colistin.

Table 1. Lipids composition in liposome formulations and their main physicochemical properties (26) (29).

Phospholipid	Chemical structure	Molecular weight (g/mol)	Phase transition temperature T_c (°C)
Cholesterol		386.664	Very high (29)
POPC (1-palmitoyl-2-oleoyl-glycero-3-phosphocholine)		760.076	-2 (26)
Sphingomyelin		710.965	38 (26)
DPSA-Na (1,2-Distearoyl-sn-glycero-3-phosphate, sodium salt)		726.99	75 (26)

2. Materials and Methods

2.1 Materials

Phospholipids used for liposome preparation, Sphingomyelin, POPC and DSPA-Na were purchased from Coatsome®. Cholesterol (from lanolin, ≥99.0%), Colistin sulfate (Polymyxin E), Ninhydrin reagent, sodium acetate trihydrate, hydrindantin, Dulbecco's Phosphate Buffer Saline (PBS) (Modified with calcium chloride and magnesium chloride), Triton™ X-100 nonionic surfactant, Mueller-Hinton (MH) broth and organic solvents (chloroform and methanol) are all purchased from Sigma-Aldrich. Fluorescent dye DiD (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine, 4-Chlorobenzenesulfonate Salt) to label liposomes for fluorescence microscopy measurements was purchased from ThermoFisher.

Bacteria strains XL1 blue (Active Ingredient resistant), K12 mVenus (Active Ingredient resistant), MG1655, L5 (Active Ingredient resistant) and L9 (Active Ingredient resistant) strains of *E. coli* were used in the experiments. *Salmonella enterica* subsp. *enterica* serovar Typhimurium SL1344 DsRed2 (Active Ingredient resistant) strain was used for *Salmonella* in the experiments.

2.2 Preparation of liposome formulations

In this work, liposomes containing colistin were formulated using Cholesterol, POPC and SM. Additionally, liposomes containing colistin with DSPA-Na (PS) phospholipid were also formulated.

Liposomes were prepared by thin film hydration method. 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), Cholesterol, and Sphingomyelin (SM) were dissolved to a final concentration of 1.0 mg/ml stocks in chloroform for POPC and Cholesterol, whereas the 1.0 mg/ml SM stock was prepared in 10% methanol, 90% chloroform. Phospholipid fluorescent dye DiD (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine, 4-Chlorobenzenesulfonate Salt), contained in all formulations, was solvated in chloroform as a 0.1 mg/ml stock. 1 mg/ml stock of colistin sulfate was prepared in equal volumes of chloroform and methanol. Formulations were prepared by mixing the required volumes stock solutions in a 50 mL round bottom flask according to colistin loading (%) and total lipids molar ratio (%) in Annex Table A.1. Formulations were left in fumehood overnight to vent, then further dried under nitrogen stream for dry film formation. The dry film was hydrated with 1 ml Phosphate Buffer Saline (PBS). After hydration, liposomes were sonicated for 10 min in an ultrasonic bath. Each liposome suspension was extruded through an Avanti Mini Extruder holding 100 nm filters (Whatman Nucleopore) 21 times and some formulations with Jacketed Liposome Extruder (Genizer) (extrusion done by compressed nitrogen with pressures up to 1.500 psi with one passage) in order to produce 1 ml 10 mM (total lipid concentration) liposome formulation. All formulations were kept at 4°C in the refrigerator. For DSPA-Na liposomes 20 % molar ratio of DSPA-Na was used for liposome formulation.

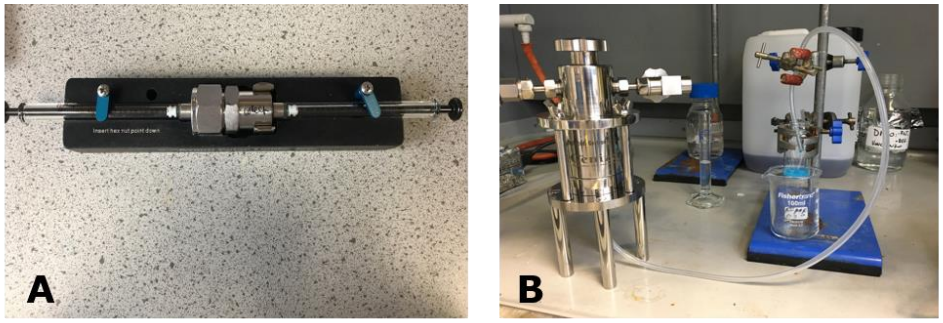


Figure 4 A) AvantiPolar Mini Extruder B) High pressure jacketed liposome extruder (Genizer)

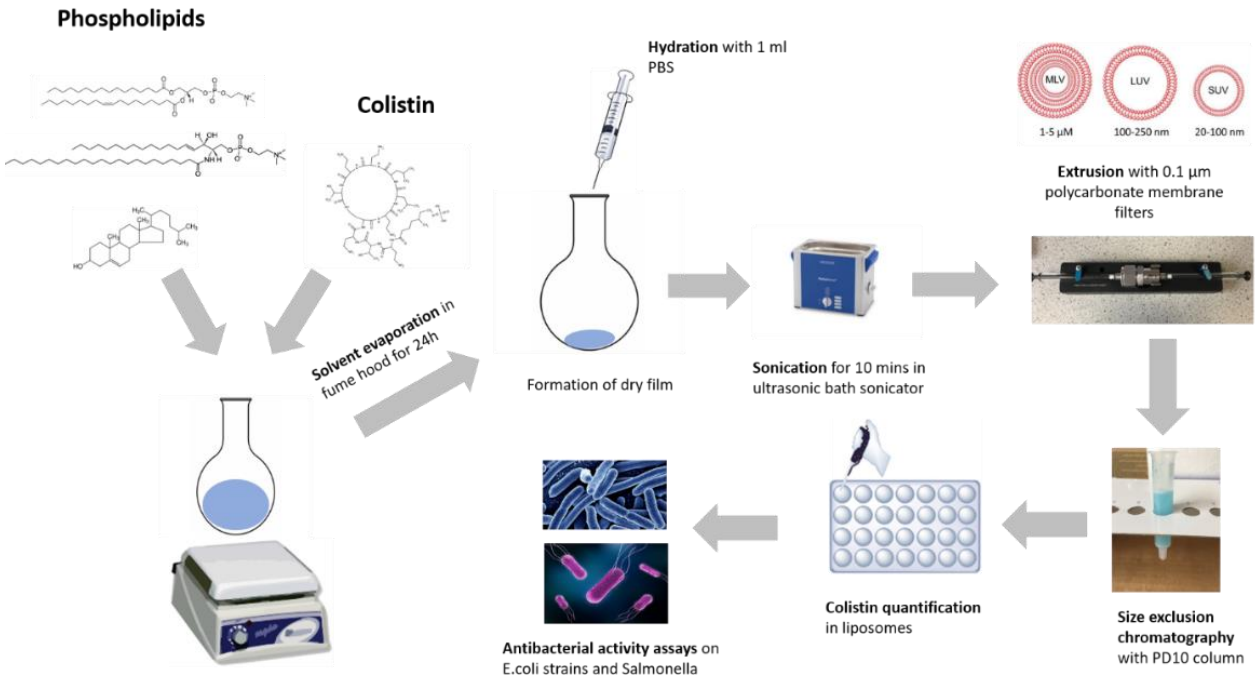


Figure 5 Liposome preparation and biological assays scheme

2.3 Liposome purification by PD10 column

Separation of unencapsulated (free) drugs from liposomes was carried out by size exclusion chromatography in PD-10 column. The resulting liposome suspensions were passed through a PD-10 Desalting Column Filter (GE Healthcare) according to the gravity protocol, with PBS as washing buffer. 12 fractions from the column were collected in eppendorfs as 300 µl volume each. The visual inspection indicates that liposomes (DiD labelled and appearing blue) are eluting from the column in 12 fractions. The first 3 fractions are observed to be blue in colour which indicates that these fractions contain liposomes (as confirmed by DLS measurements), later fractions appear significantly less blue in colour, again indicating low content of liposomes. Free colistin retention time was measured in the same condition of liposomes purification process. Free colistin appeared from fraction 4, therefore colistin-liposome fraction 2 or fraction 3 were used for the experiments presented here.

2.4 Size and zeta potential of liposomes

Size distribution and zeta potential of liposomes were measured with Dynamic Light Scattering (DLS) technique using a Zetasizer NanoZS (Malvern Panalytical). Samples were diluted as 1:100 in milli-Q water. Scattering intensity and particle size were determined at attenuation 8.

2.5 Colistin quantification in liposomes

Ninhydrin assay was carried out for the determination of colistin concentration (µg/ml). 4 M sodium acetate buffer was prepared by dissolving 54.4 g sodium acetate trihydrate in 50 ml of milli-Q water in a 100 ml beaker. Glacial acetic acid was added dropwise to bring pH of the solution to 5.2 and milli-Q water was added up reach 100 mL final volume. Ninhydrin agent was prepared by addition of 0.2 g ninhydrin and 0.03 g of hydrindantin in a scintillation vial. 7.5 ml DMSO and 2.5 ml sodium acetate buffer were added to the vial. Ninhydrin agent was kept protected from light by covering the vial with aluminium foil. 1 mg/ml colistin sulfate stock solution was prepared in PBS. Serial dilution was made in 96 well plates with a multichannel pipette. Liposome samples were diluted as 1:5 dilution in PBS for measurements. 75 µl of ninhydrin agent was added to all wells. Plates were incubated at 80 ˚C for 30 minutes in a water bath. 100 µl stabilising solution (1:1 isopropanol- milli-Q water) was added to all wells. Absorbance was measured at 570 nm by a microplate reader. The calibration curve was established with known concentrations of colistin (x-axis) (500 ug/ml-0.48 ug/ml) with absorbance values (y-axis) obtained from microplate reader (TECAN Plate Reader) to calculate the concentration of encapsulated colistin in liposomes. Calibration curve equation is found as $Y= 0.004378 X + 0.1675$, $R^2 = 0.9987$.

Fluorescence emission of DID in pre and after column liposomes was used to determine the total lipid concentration of the fractions after column purification. Samples were diluted 10% in 0.2% v/v Triton X-100 in PBS and measured for DiD fluorescence intensity (Tecan Plate Reader, $\lambda_{excitation}$ 610 nm, $\lambda_{emission}$ 670 nm).

Colistin concentration for each fraction and encapsulation efficiency was calculated as follows;

Total lipid concentration in fraction (mM) = Fluorescence intensity after column × 10 mM / Fluorescence intensity pre column

Colistin concentration in liposomes was normalised according to the total lipid concentration in each fraction in order to calculate drug encapsulation efficiency.

Encapsulation efficiency (%) = (Normalised experimental loading / Theoretical loading) × 100

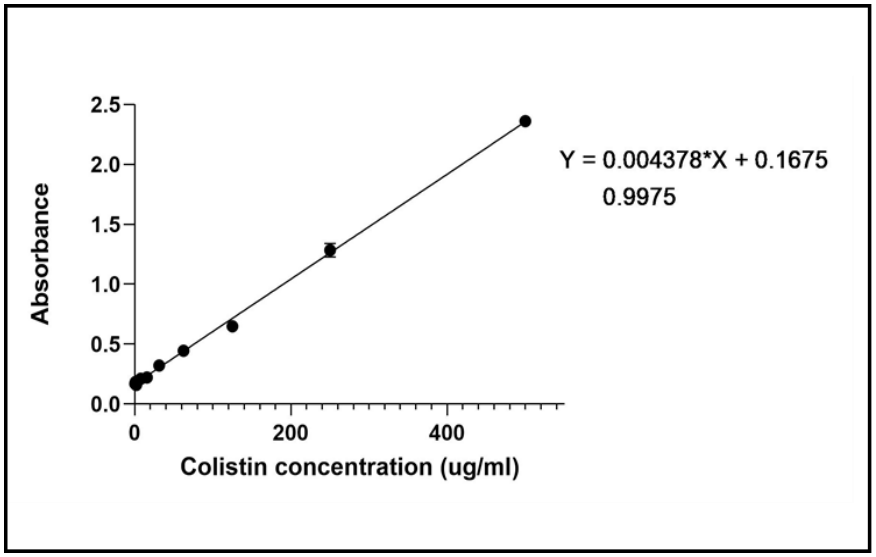


Figure 6 Calibration curves for colistin

2.6 Biological tests

2.6.1 Antibacterial activity assay by measurement of optical density

MH growth medium was prepared by dissolving 0.21 g of MH broth powder in 100 ml Milli-Q water. The twice concentrated MH broth was prepared for dilution of the bacteria suspension with liposome suspensions in measurements. *E. coli* strains and *Salmonella* were cultured and made available for the experiments presented in this work by Dr Rosa Catania. A single colony of each bacterium was suspended in MH broth (4 ml) and incubated at 37°C, shaking at 245 rpm overnight. Aliquot of overnight bacterial suspension was diluted in MH broth (2x), optical density of this bacteria suspension was adjusted to 0.03 AU. 100 µl aliquots of these cell suspensions were mixed with 100 µl of PBS containing different concentrations (1 µg/ml, 0.5 µg/ml, 0.25 µg/ml, 0.1 µg/ml) of free colistin, or equivalent colistin-liposomes in a 96 well plate. The growth curves of bacteria strains were determined in 3 independent replicate wells for each condition. MH broth (growth medium) with PBS was used as negative control and Active Ingredient was used as a positive control in these experiments. Growth of bacteria strains with free drug and liposome formulations were analysed by optical density at 600 nm during over 12 hours at 37°C in a shaker (245 rpm) Epoch 2 microplate reader (BioTek).

2.6.2 Antibacterial activity test by time-kill assay

In vitro growth of bacteria population within specified sampling times after exposure to liposomes and free colistin was measured which is expressed as killing curves. CFU·ml^{-1} (Colony forming unit) was found by counting bacteria colonies on agar plates obtained from incubation of *E. coli* MG1655 bacteria strain with free colistin, 10 % colistin and PBS in each time points for 6 hours duration. Initially, optimum bacteria dilution found as 1:20000 which corresponds to 10^4 CFU/ml as initial colony growth. Agar plates prepared by melting MH agar and pouring 15 ml in petri-dish plate. Stock solutions for 10 % liposome and free colistin were prepared by 2 step dilution to obtain 1 µg/ml concentration. Bacteria dilution was done by addition of 5 µl overnight bacteria culture to 1ml of MH broth then from this 100 µl was taken to 10 ml of MH broth. 100 µl of free colistin and colistin loaded liposome were put in the first row of 96 well plates with 100 µl of bacteria suspension in MH broth (repeated for total 8 plates, each plate representing one-time point). Plates were incubated at 37°C, shaking at 245 rpm. After incubation, 1: 10 serial dilutions were done in plates from B to H rows. 10 µl from each well was plated on MH agar plates. Plates were incubated overnight at 37°C to allow formation of colonies. According to countable colonies position on plates dilution factor changes. -1 (Dilution factor: 10^1), -2 (Dilution factor:10^2), until -7 (Dilution factor: 10^7). The following day, colonies were counted and CFU·ml^{-1} was calculated as follows;

$$\textbf{CFU} \cdot \textbf{ml}^{-1} = \text{Number of colonies counted} \times \text{Dilution factor}$$

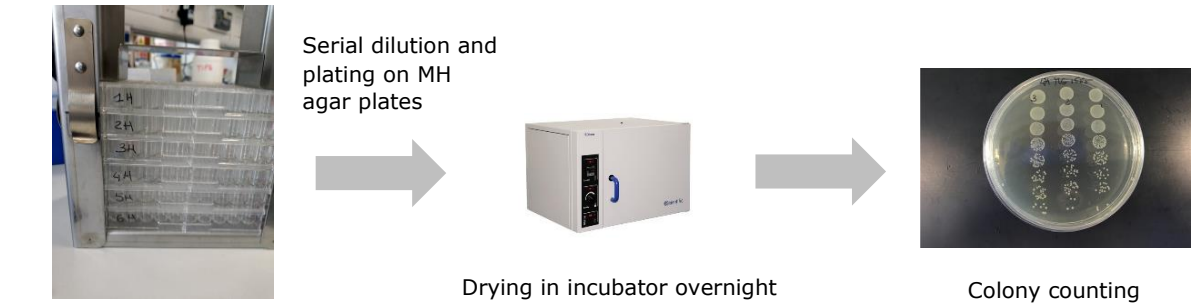


Figure 7 Time-kill test scheme

2.7 Statistical tests

All measurements were performed in triplicate and resulting data are presented as the arithmetic mean of the repeats unless stated otherwise. Error bars represent the standard deviation of the mean. For multiple comparisons at a single time point or concentration, data were analysed using either one-way analysis of variance (ANOVA) for parametric data The appropriate post-hoc test was carried out to correct for multiple comparisons with the corresponding p-values are reported in results. All statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software Inc., USA). Significance limit was set at 0.05.

3. Results

We investigated two groups of liposome formulations, reported in Table 2. Firstly, we investigated the size and zeta potential of colistin encapsulated liposomes and colistin content in liposomes. Their antibacterial activity was measured by broth microdiluition assay. Secondly, liposome containing DSPA-Na were prepared and characterised, due to time constraints the antibacterial activity of these set of formulations was not tested.

Fınally, we explored a combination therapy system by co-administration of tetraycline and colistin in liposome.

Table 2 Liposome formulations with different drug loading (%) and lipid composition (All formulations contain Cholesterol, POPC and SM)

Formulation	DSPA-Na (PS) lipid	Colistin
Colistin liposomes		
0%		
1%		✓
2.5%		✓
5%		✓
7.5%		✓
10%		✓
PS liposomes		
20PS – 0%	✓	
20PS –10%	✓	✓

3.1 Liposome characterisation

3.1.1 Size and zeta potential of liposomes

Colistin loaded liposomes in all formulations except 10 % formulation were in the size range of 120-150 nm of nm with PDI less than 0.2, showing relatively narrow size distribution as shown in Fig. 8. 10 % formulation size was 255 ± 14 nm with PDI 0.36 and 10 % intensity of peak was observed with 3593 nm size.

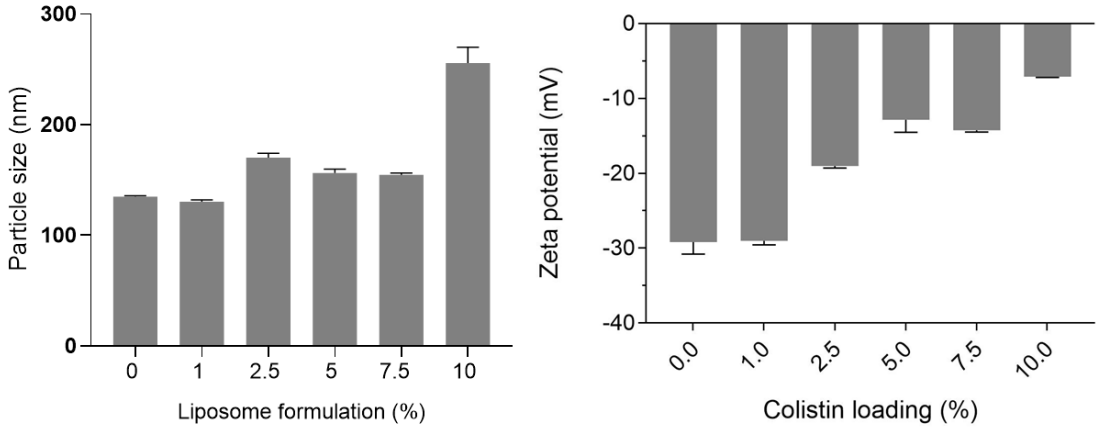


Figure 8 Colistin encapsulated liposomes size and zeta potential

Colistin liposomes (0% and 10%) extruded with Jacketed Liposome Extruder produced 165.3 ± 2.1 nm with PDI 0.157 for 0 % formulation and 153.2 ± 0.7 nm with PDI 0.2 indicating this extrusion can produce liposomes with 100 nm range and with narrow size distribution. Significant increase of zeta potential to positive charge was observed with increasing colistin loading in colistin encapsulated liposomes, which indicates incorporation of colistin in liposomes because of its cationic ring. 10 % colistin formulation had -7.09 ± 0.08 mV zeta potential. Negative zeta potential indicates good electrostatic stabilisation of liposome formulations.

DSPA-Na (PS) liposomes were prepared for future investigation of liposome-bacteria (*E. coli and Salmonella*) interaction depending on the change of surface charge. PS liposome without colistin zeta potential was -49.93 ± 1.08 mV indicating negatively charged liposomes and 10 % colistin liposomes zeta potential was increased to -25.7 ± 0.9 mV. DSPA-Na liposomes (0% and 10% colistin loading).

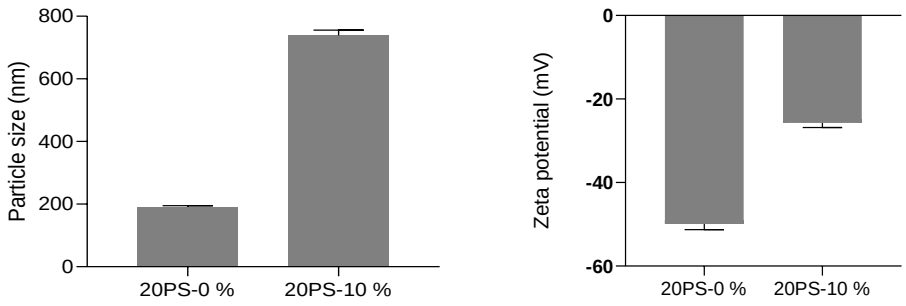


Figure 9 Colistin encapsulated PS liposomes size and zeta potential

3.2 Colistin quantification

The lowest detection limit of colistin in liposome formulations were found to be 1 µg/ml (31). Detection in fractions 1 and 2 could be not precise because colistin amount is very low in these fractions. In later fractions colistin concentrations reach up to 125 µg/ml. The measurement suggested that free (not entrapped) colistin is eluting from column after fraction 4. In addition, theoretical and experimental drug loading were close indicating colistin can efficiently be incorporated into liposomes. Colistin encapsulation was high due to colistin presence in bilayer and not in the core of liposomes.

Table 3 Colistin loading and encapsulation efficiency in Cholesterol, POPC, SM liposomes with increasing drug loading

Formulation	Colistin concentration (Experimental loading) (µg/ml)	Theoretical loading (µg/ml)	Encapsulation efficiency (%)
1 %	13.5 ± 0.934	12.68	106.47 ± 7.368
2.5 %	25.60 ± 0.929	31.69	80.78 ± 2.932
5 %	55.80 ± 2.892	63.38	88.04 ± 4.564
7.5 %	69.60 ± 0.868	95.07	73.21 ± 0.913
10 %	111.0 ± 3.842	126.76	87.57 ± 3.031

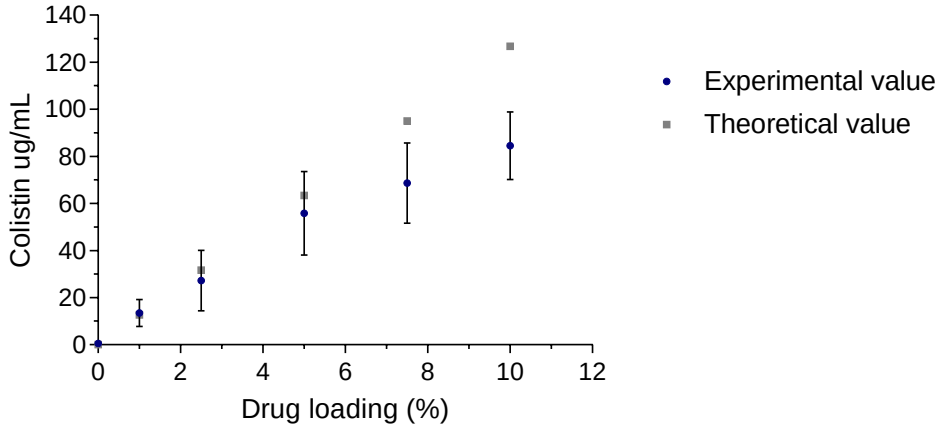


Figure 10 Colistin encapsulated liposomes experimental and theoretical drug loading

It was concluded that encapsulation efficiency is similar in all formulations. 10 % colistin encapsulated formulation had the highest colistin concentration.

3.3 Biological assays

3.3.1 Antibacterial activity test by measurement of optical density

Growth curves reveal that the inhibition of bacterial growth was enhanced by colistin loaded liposomes (10 % colistin laoded formulation) relative to the equivalent concentration (0.5 µg/ml) of free colistin. In fig. 11, free colistin and liposomal colistin formulations comparison is plotted. The concentration was chosen as 0.5 µg/ml, below MIC of free colistin, in order to understand if colistin encapsulated liposome can decrease minimum inhibitory concentration of colistin which is 1 µg/ml. 10 % liposome formulation significantly inhibits growth of *E. coli* L5 and L9 compared to equivalent concentration of free colistin. ($p<0.0001$) In addition, 10 % liposome formulation inhibits growth significantly more than other liposome formulations. ($p<0.0001$).

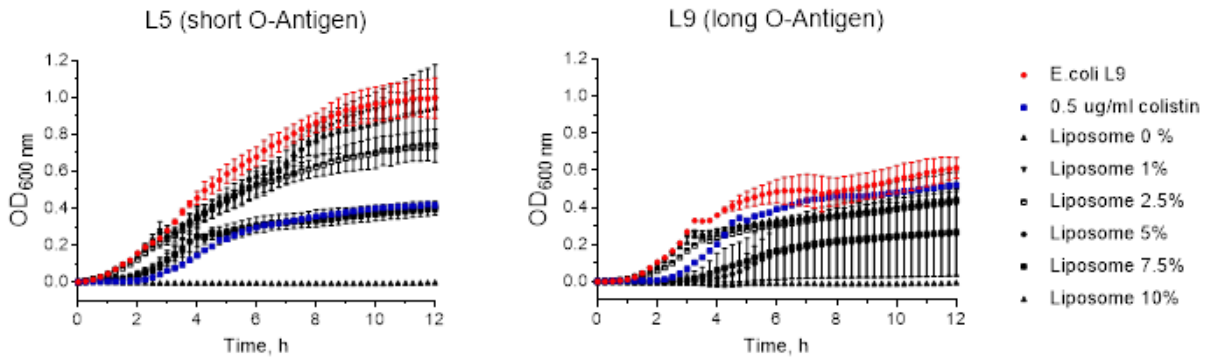


Figure 11 Shaker Plate Optical Density (OD, 600 nm) measurement of *E. Coli* strains (L5 and L9) in broth culture at 37^0 C over 12 hr with concentration of 0.5 µg/ml. Bacteria growth curve shown as (red) and free colistin as (blue). The percentage figure refers to the molar concentration of colistin in liposomal formulation, whereas the figure in µg/ml refers either to a known concentration of colistin, or a dilution of the liposomal formulation based on the amount of colistin determined to be in the fraction through Ninhydrin assay. Optical density are shown as the mean of 3 independent measurements, for which error bars represented.

Fig. 12 shows that 0.5 µg/ml free colistin and 10 % liposome formulation have no significant effect on inhibiting growth of Salmonella. It is hypothesized that long O-antigen chains on outer membrane prevent colistin to interact with bacteria and to show its antibacterial effect, suggesting that the long O-antigen masks anionic sites on

lipopolysaccharide. 10 % liposome formulation inhibits growth of *E. coli* XL1 and E. coli K12 significantly more than free colistin ($p<0.0001$) indicating 10 % liposome formulation is effective against XL1 and K12 strain.

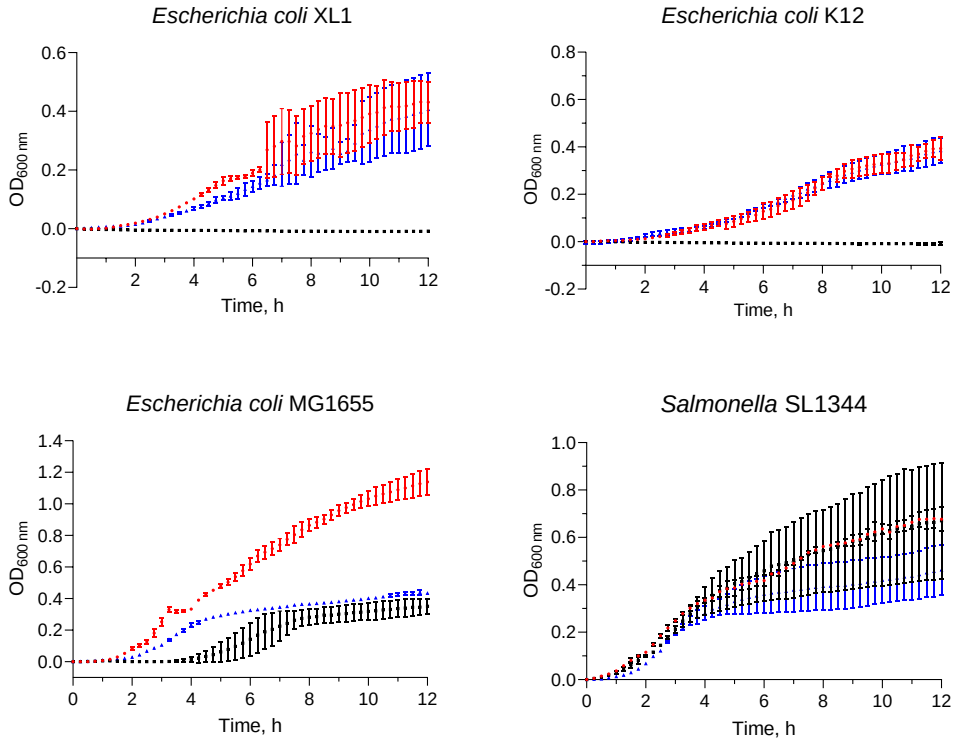


Figure 12 Shaker Plate Optical Density (OD, 600 nm) measurement of E. coli strains and Salmonella (SL1344) in broth culture with 10 % colistin liposome and free colistin equivalent concentrations (0.5 µg/ml). Bacteria (red), Liposome 10 % (black) and 0.5 µg/ml colistin (blue).

3.3.2 Antibacterial activity test by time-kill assay

Colistin encapsulated liposome formulations were tested for bacteriostatic effect for 4 hours. Firstly, we investigated the concentration of bacteria in suspension by serial dilution method as shown in Fig. 13 *E. coli* MG1655 colonies amount increased with the increasing incubation time indicating bacteria growth increase during 4 hours.

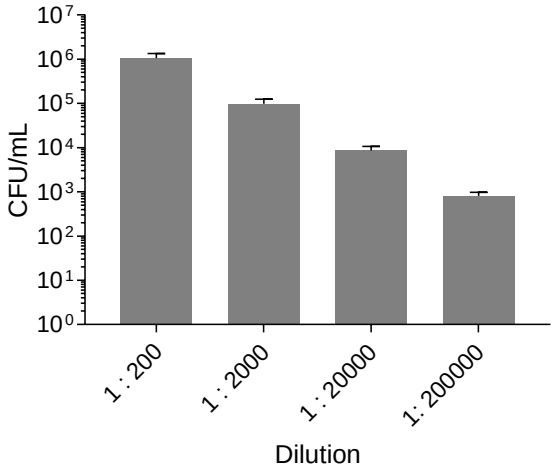


Figure 13 Bacteria dilution for time-kill assay with *E. coli* MG1655

Colistin liposome showed significant decrease in bacteria colonies after 3 hours, however free colistin did not show a constant profile of decrease in bacteria colonies according to Fig 17. After 3 hours incubation, bacteria colonies increased in colistin loaded liposome plate slightly. Growth curves (Fig. 12) and killing curves (Fig. 14) show similar findings for colistin liposome (10%) and free colistin has same bacteria growth (*E. coli* MG1655) in both experiments in 4 hours duration. *E. coli* MG1655 growth was as same as previous experiments with time-kill assay which proves the reproducibility of bacteria growth. Bactericidial effect test proved that colistin liposome formulation was efficient because this test gives direct indication of viable bacteria cells.

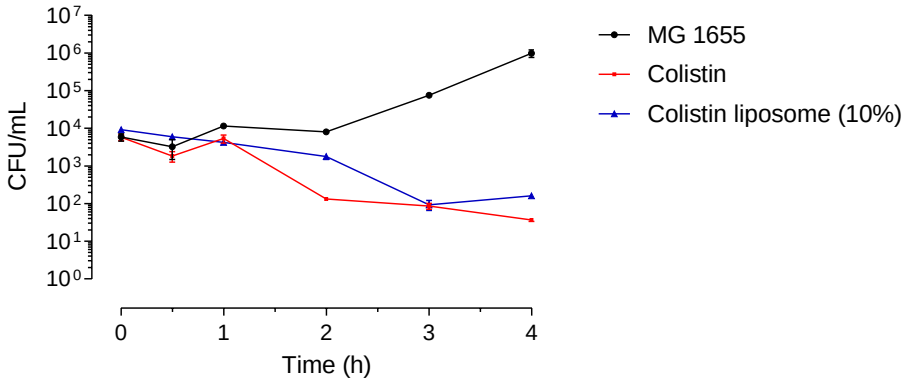


Figure 14 Killing curves for *E. coli* MG1655, free colistin and colistin liposome (10%) at the same concentration (0.5 µg/ml)

4. Discussion

In the present study, the obtained results suggest that liposomal colistin with highest drug loading (10 %) improves the antimicrobial effect compared to free colistin, exhibit specific targeting to E. coli strains, the use of liposomes enhance MIC (Minimum inhibitory concentration) of colistin to less than 1 µg/ml (sub-MIC of colistin) resulting in maximising

safety. In addition, these colistin-liposomes are currently investigated as targeting agent for combination therapy approach. The growth curves results for colistin liposomes and free colistin demonstrates that liposomal colistin (10 %) completely inhibits growth of *E. coli* at 0.5 µg/ml concentration which can be explained by concentration dependent effect of liposome on bacteria. We can hypothesize that increased colistin amount in liposomes leads to high local drug concentration at bacteria outer membrane facilitating disruption of outer membrane more than free colistin and also allows colistin to show antibacterial effect in the inner membrane. According to the obtained results, the mechanism of action of liposomes on bacteria is thought to be colistin liposome releasing high load of colistin at the site of action to exert its effect which can be called as "concentration effect". Membrane disruption and concentration effect of colistin loaded liposomes together enhance antibacterial activity. Encapsulation of antibacterial agent in liposomes increases permeability of bacteria outer membrane which enables higher uptake of colistin inside bacteria cells.

Growth curves of different *E. coli* strains and *Salmonella* indicates that growth inhibition phenomenon by colistin loaded liposomes does not occur for *Salmonella* which can be attributed to subtle variations in LPS structure, such as longer O-antigen chains expressed by *Salmonella* confirming that LPS structure has an important effect on determining binding affinity of liposomes. Additionally, colistin liposomes build electrostatic and hydrophobic interactions with LPS in order to exert its bactericidial effect.

Liposome physicochemical properties and lipid composition have a strong impact on encapsulation efficiency and antibacterial activity regarding their surface charge, bilayer rigidity, size, collodial stability, phase transition temperature of phospholipids during liposome preparation (3). Fatty acid chain length increase in phospholipids, increases drug encapsulation in liposomes (32). For this liposome formulation containing Cholesterol, POPC and SM rigidity of bilayer is high due to Cholesterol and SM presence which also helps to increase entrapment efficiency of the drug and prevent leakage by providing further stability in blood circulation (33). In addition, POPC makes bilayer more fluid and permeable due to its -2 ˚C phase transition temperature and unsaturated fatty acid chains (33). The oleoyl side chain in POPC would allow for better packing due to its greater conformational freedom imparted by the double bond present in the chain (33). Since colistin charge is implicated in LPS binding, charge characteristics of liposome formulations directly affect antibacterial activity.

Independent tests of growth curves showed similar results and *E. coli* growth followed same profile for time-kill antibacterial activity tests which supports that the data obtained is reproducible by different experiment.

5. Conclusion and perspectives

In this study, enhanced antibacterial effect of colistin against 5 distinct *E. coli* strains (MG1655, XL1, K12, L5 and L9) was achieved by colistin containing liposome formulations. These liposome formulations were considered as a model system to repurpose a last resort antibiotic and enhance antibacterial activity offering promising results for treatment of gastrointestinal infections. Furthermore, it was demonstrated that antibacterial activity is enhanced with high drug loading of liposomal colistin compared to free colistin. Colistin loaded liposomes can deliver sufficient drug to bacteria cell membrane achieving high local drug concentration effect which other antibacterial agents in their free form can not achieve. Colistin loaded liposomes were produced with 120-150 nm size with range. Highest encapsulation efficiency obtained was for 10 % colistin encapsulated liposome formulation with 87.57 ± 3.031 encapsulation efficiency (%). These findings open novel pathways towards the selective targeting of Gram-negative bacteria in research and in the clinic. These liposomal carriers are very promising for selective targeting of Gram-negative bacteria compared with classes of antibiotics that do not ordinarily permeate through Gram-negative bacteria outer membrane.

Although these nanocarriers are effective in killing *E. coli* strains, works are undergoing in Prof. Stolnik's group to search for the implications of these findings such as, testing Active Ingredient and colistin encapsulated formulations on other *E. coli* strains, visualisation of liposome-bacteria interactions by advanced microscopic techniques in order to understand mechanism of action better, mucopenetration studies by Caco-2 cells in order to evaluate liposomes penetration through mucus barrier in order to target *E. coli* in gastrointestinal tract, cytotoxicity studies in order to prove colistin loaded liposomes decrease the toxicity compared with the free colistin and to reach higher drug loading active loading methods could be implemented while preparing liposome.

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Annex

Table A.1 Composition of liposome formulations. Numbers expressed *as percent molar ratio* (%). (SM: Sphingomyelin)

Formulation	POPC	Cholesterol	SM	DSPA-Na	DiD	Colistin
Cholesterol + POPC + SM liposomes						
0 % (Control)	45.25	40.00	14.55	0	0.2	0
1 %	44.25	40.00	14.55	0	0.2	1.0
2.5 %	42.75	40.00	14.55	0	0.2	2.5
5 %	40.25	40.00	14.55	0	0.2	5.0
7.5 %	37.75	40.00	14.55	0	0.2	7.5
10 %	35.25	40.00	14.55	0	0.2	10.0
DSPA-Na liposomes						
0 %	25.25	40.00	14.55	20.0	0.2	0
10 %	15.25	40.00	14.55	20.0	0.2	10

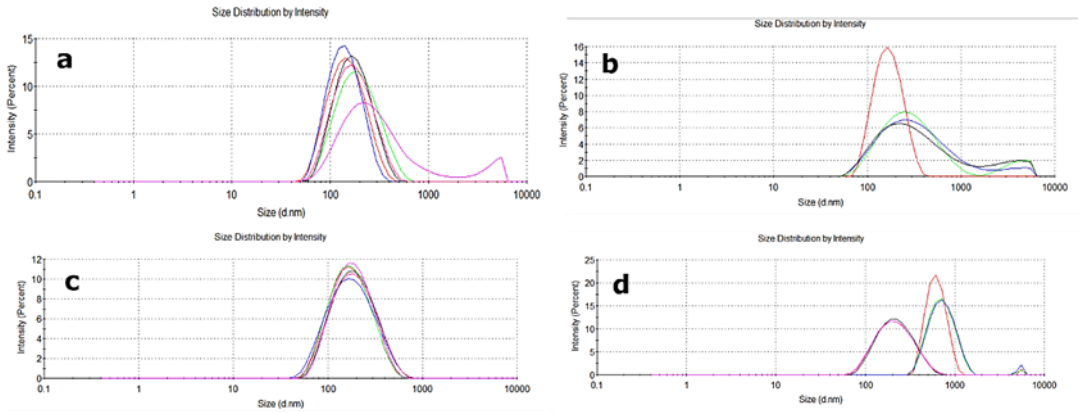

Figure A.2 Particle size (z-average hydrodynamic diameter) and particle size distribution of liposome formulations. In **(a)** size results of colistin loaded liposomes, in **(b)** size results of Active Ingredient and colistin loaded liposomes, in **(c)** size results of colistin liposomes (0% and 10% formulation) extruded with Jacketed Liposome Extruder (Genizer), in **(d)** size results of DSPA-Na negatively charged liposomes are shown.